PERCEPTION AND IMPLEMENTATION OF PCR PROTOCOL AT KARACHI BASED INSTITUTIONAL LABORATORIES - KAP STUDY

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ABSTRACT

Objectives: 1) To shed light on the perception and practices of certified lab professionals regarding PCR protocol implementation. 2) To decipher the association of perception with implementation of PCR protocol. 3) To provide a review of the steps of qualitative PCR based on the current guidelines.

Study design: Cross-sectional survey based study (KAP)

Settings: Non-probability convenience sampling was used to collect data from qualified lab professionals working at various Karachi-based diagnostic / research facilities equipped with PCR.

Materials and Methods: The method of data collection was mixed (quantitative and qualitative). A self-structured questionnaire comprising 18 questions on PCR thermal cycles, DNA polymerase, PCR primers, DNA extraction methods, gel electrophoresis and lab environment etc. was disseminated among participants. Measure of central tendency, frequency distribution, chi-square test of independence and thematic analysis were performed.

Results: The mean temperatures chosen for annealing, denaturation and extension phases of PCR cycle were 55°C, 93°C and 71°C. The mean thickness of agarose gel and the mean size of DNA ladder chosen for gel electrophoresis was 4 mm and 127 bp**.** PCR performance showed a statistically significant association with the decision to aim for DNA high purity standards (p 0.008). The decision to follow the manufacturer's protocol had a very significant association with the decision to alter cycle temperature (p 0.001). Thematic analysis revealed the hindrances in construction of a PCR based laboratory, the preferred manufacturers of PCR kit, and the preferred lab sterility methods.

Conclusion: We discerned the preferences and practices of lab professionals for PCR. We also elucidated the working of qualitative PCR in light of internationally accepted guidelines.

Keywords: Base Composition, DNA, Complementary, DNA Primers, Laboratories, Nucleic Acid Denaturation, Polymerase Chain Reaction.

INTRODUCTION

Kary Mullis invented the polymerase chain reaction (PCR) technique for which he won the Nobel prize in Chemistry. The advent of PCR in 1985 has made it an invaluable technique in scientific research and clinical investigation.[1] Its application in medical & allied fields, for instance, in gene expression studies, detection of mutations, pathogen detection, forensic analysis and targeted next generation sequencing, has been associated with a paradigm shift in our understanding of biology and pathology.[2] PCR embodies the principle of detection and amplification of a single specific molecule of DNA into billions of copies. Therefore, PCR serves as an indispensable tool in the pursuit to achieve a better understanding of the complex biological and pathological processes which trickle-downs to better bespoke clinical care and disease management. A conventional PCR is agile, reliable, easy to operate, and economical.[3]

The current PCR technology can amplify a minuscule amount of nucleic acid sequence. The PCR uses an in-vitro method of replicating the DNA. This process is based on three main steps which are thermally regulated, i.e., denaturation, annealing, and extension.[4]

The aforementioned steps usually last 30-40 cycles, although the number of cycles might vary based on the quantity of DNA and the required yield of PCR product. The ongoing cycles exponentially amplify the resulting PCR product, thereby generating copies in billions. The

amplification reaction in PCR can be divided into three phases with respect to time. The phases are "exponential amplification", "linear phase", and "plateau phase".[5]

There are six basic constituents of a standard PCR reaction. These include the DNA template material that has to be amplified, a pair of primers (forward and reverse), deoxynucleoside triphosphates (dNTPs), thermostable DNA polymerase (commonly, Taq or Pfu), MgCl2 ions and buffer solution.[6] The function of the primers is to determine the region of DNA that needs to be amplified. The final PCR product is obtained when the DNA polymerase reacts with dNTPs.[7]

The DNA replication phase in PCR is dependent upon the DNA polymerase. Together with the magnesium ion, DNA polymerase acts as an impetus by adding nucleoside monophosphates to the amplifying DNA strand.[8]

A favorable PCR result is greatly dependent upon following the proper rules. Some of the common guidelines posit using both dedicated and calibrated pipettes, working in a dedicated workspace, and cleaning the benchtop with ethanol etc. These, along with other instructions in this article, can be collectively termed as "good PCR practices".[6]

In addition to the conventional (qualitative or standard) PCR, there are numerous specialized variants of PCR, such as quantitative PCR (qPCR alias real-timePCR), reverse transcriptase PCR (RT-PCR), qualitative reverse transcriptase PCR (qRT-PCR), nested PCR, multiplex PCR, hot start PCR, touchdown PCR, colony PCR, assembly PCR, asymmetric PCR, nanoPCR, allele-specific PCR, methylation-specific PCR and in-situ PCR. [9, 10]

Real-time PCR allows the measurement of the PCR product in real-time. The exponential phase of the PCR is highly sensitive and specific. During this time, there is a two-fold increase in the PCR products. Real-time PCR enables the assessment of PCR products during this phase.[5]

Till date, numerous studies on conventional PCR have been conducted. Some studies have explained the methodology of PCR, while others have shown the application of PCR in various fields of science. Nevertheless, none of these studies attempted to discern the perception and practice of PCR protocol implementation by certified lab professionals such as pathologist, molecular biologist, microbiologist, biochemist, biotechnologist and geneticist in healthcare facilities; so as to decipher and highlight the common and best practices with regards to this extremely important but esoteric laboratory technique. Therefore, the aim of this study is to conduct a survey among qualified lab professionals to determine their attitude regarding usage of PCR protocol and their practice encompassing all the steps of PCR workflow. This will abet the healthcare faculty and the students by providing them a summary of PCR workflow practiced by experts in their respective fields. The objectives of this study are to: 1) highlight the perception and practices of lab professionals with regards to implementation of PCR protocol; 2) decipher the relationship of perception of lab professionals with regards to PCR usage and implementation of PCR protocol; 3) provide a review of the basic steps of the standard PCR procedure based on the current up-to-date guidelines. Our findings will abet a professional in pursuing a "good PCR practice".

The study is the first of its kind in which researchers will evaluate the perception and the practices of lab professionals at both public and private healthcare facilities of Karachi with regard to the implementation of PCR.

MATERIALS AND METHODS

Study design: cross-sectional survey based KAP study

Study site: *R*esearch institutes and diagnostic facilities which incorporate laboratory based molecular techniques for diagnostic and academia / research purposes (Dow University of Health Sciences, Karachi University, Pakistan Council of Scientific & Industrial Research, Ziauddin Medical University, and Bahria Medical University).

Study participants: Qualified lab professionals who routinely perform laboratory based molecular techniques.

Sampling technique: Non-probability convenience based sampling technique. We chose this technique because we were granted permission to collect data of lab professionals from the aforementioned academic institutes by their respective lab managers. Therefore, our final sample size was 79.

Inclusion criteria: Lab professionals entailing teaching / non-teaching faculty members and graduate students.

Exclusion criteria: 1) lab technicians 2) faculty and graduate students who have no prior knowledge of PCR

Data Collection Method: A self-constructed and self-administered questionnaire was distributed amongst the respective participants. The questionnaire attempted to evaluate the working knowledge of PCR among the participants of this study. It comprised eighteen questions distributed into four categories. The questions comprised "yes" and "no" options. Some of these questions entailed sub-questions in which the participants were required to express their opinion

(open ended, qualitative questions). All of the questions were based on the implementation and perception of PCR protocols and the institute-based implication of these practices.

An informed consent was obtained by the research members prior to the dissemination of the questionnaire among the participants. Names were not asked in the questionnaire. These two steps were taken to ensure confidentiality of the participants.

RESULTS

Our study comprised 79 participants. The mean temperature (with SD) set by participants for annealing, denaturation and extension stages of PCR cycle were 55(±4.6)°C, 93(±6.4)°C and 71(±5.4)°C, respectively. The mean thickness of the agarose gel and the mean size of the DNA ladder set by the participants for agarose gel electrophoresis was 4 mm and 127 bp, respectively**.** While, the mean number of wells utilized for the gel run were 20. Moreover, majority of the participants (n=52) stated that the conventional PCR was unable to cater the needs of some laboratory analysis. This implies that other specialized PCRs would be necessary for other specialized lab work. Purity of DNA samples was another decisive factor for obtaining good results as noted in the filled questionnaires (n=70). It is worth noting that the majority of the participants preferred to use the commercially available primers (n=60) rather than opting to design the primers themselves. For further reference, measure of central tendency (mean and standard deviation) of all the variables are mentioned in Table 1 and Table 2 respectively.

Cross tabulation of qualitative variables via univariate analysis revealed that PCR performance had a statistically significant association with the decision to aim for high purity standards in DNA (p 0.008). The decision to follow the manufacturer's protocol had a very significant association with the decision to alter the temperature of PCR cycles (p 0.001). Table 3 shows a list of

qualitative variables analyzed by univariate test. Thematic analysis revealed that lack of funds was considered by some participants (n = 21) as the main hindrance in construction of a laboratory containing PCR. Some participants (n = 23) stated that sterility of their laboratory is achieved by using Ethanol. Qiagen was the most common (n=20) supplier chosen for PCR kits by the participants of our study. Detailed thematic analysis of qualitative questions is given in Table 4.

Table 1: Measure of central tendency of quantitative variables

		PCR annealing temp	PCR denaturation temp	PCR extension temp	What's the thickness of gel which u utilize	How many wells do u use to load samples	What is the size of ur DNA ladder
N	Valid	76	76	76	78	78	78
	Missing	4	4	4	2	2	2
Mean		55.34	93.12	71.51	4.19	20.17	127.09
Std. Deviation		4.629	6.477	5.430	3.882	23.474	226.447

Table 2: Frequency distribution of qualitative variables

VARIABLE			
Do you follow manufacturer's protocol	**YES n (%)**	**Non (%)**	**Total n (%)**
	49 (61.3)	30 (37.5)	79 (98.8)
If not follow manufacturer's protocol, do u alter temp	**YES n (%)**	**Non (%)**	**Total n (%)**
	34 (42.5)	37 (46.3)	71 (88.8)

Did u purchase commercially available primers	**YES n (%)**	**Non (%)**	**Total n (%)**
	60 (75.0)	17 (21.3)	77 (96.3)
Do u prefer to design ur own primer	**YES n (%)**	**Non (%)**	**Total n (%)**
	34 (42.5)	42 (52.5)	76 (95.0)
Do different PCR brands performance varies in relevance to sensitivity and specificity	**YES n (%)**	**Non (%)**	**Total n (%)**
	69 (86.3)	10 (12.5)	79 (98.8)
Does conventional PCR cater all needs in lab	**YES n (%)**	**Non (%)**	**Total n (%)**
	27 (33.8)	52 (65.0)	79 (98.8)
(a) Are u using other types of NON conventional PCR	**YES n (%)**	**Non (%)**	**Total n (%)**
	56 (70.0)	19 (23.8)	75 (93.8)
(b) If Not, Do you consider the need for above mentioned PCRs in the near future	**YES n (%)**	**Non (%)**	**Total n (%)**
	44 (55.0)	28 (35.0)	72 (90.0)
Do u sometimes alter gel thickness	**YES n (%)**	**Non (%)**	**Total n (%)**
	43 (53.8)	36 (45.0)	79 (98.8)
Do u aim for high purity standards in DNA	**YES n (%)**	**Non (%)**	**Total n (%)**
	70 (87.5)	9 (11.3)	79 (98.8)
Does DNA purity affect validity of ur results	**YES n (%)**	**Non (%)**	**Total n (%)**
	65 (81.3)	14 (17.5)	79 (98.8)
Are conventional pipette still in use	**YES n (%)**	**Non (%)**	**Total n (%)**

	33 (41.3)	45 (56.3)	78 (97.5)
Are you using newer pipette with better calibration	• **YES n (%)**	**Non (%)**	**Total n (%)**
	44 (55.0)	19 (23.8)	63 (78.8)
(a) Do u use Taq Polymerase by manufacturer or commercially available	**YES n (%)**	**Non (%)**	**Total n (%)**
	67 (83.8)	6 (7.5)	73 (91.3)
(b) Does this affect the Performance of PCR	**YES n (%)**	**Non (%)**	**Total n (%)**
	62 (77.5)	11 (13.8)	73 (91.3)
Setup of lab in terms of safety and sterility	**BSL1 n (%)**	**BSL2 n (%)**	**BSL3 n (%)**
Total = 79 (98.8)	27 (.8)	40 (50.0)	12 (15.0)

Table 3: Cross-tabulation of qualitative variables by univariate analysis

Cross-tabulation of qualitative variables by univariate analysis	**Performance of PCR**	**What is the setup of your lab in terms of safety and sterility**	**Following manufacturer's protocol**
Do you aim for high purity standards in DNA	p 0.008, OR 1.49	p 0.24	
Do you use Taq Polymerase provided by the manufacturer or commercially available one	p 1.00, OR 1.02		

Are conventional pipettes still in use	p 0.75, OR 2.75		
Are you using newer pipette with better calibration	p 1.00, OR 1.75		
What is the setup of your lab in terms of safety and sterility	p 0.55		
Do you alter temperature of PCR cycles			p 0.001, OR 0.24
Do you purchase commercially available primers			p 0.57, OR 1.42
Do you prefer to design your own primers			p 0.81, OR 1.54

Table 4: Thematic analysis based on coded themes of open ended qualitative questions

How do you maintain sterility of lab environment and what steps you take for decontamination?	**What are hindrances and how feasible is it in the existing lab condition?**	**Who are your suppliers?**	**What DNA extraction methods do you use?**
Autoclave (6)	Funds (21)	Abbot (10)	organic method [phenol–chloroform] (34)
Ethanol (23)	no its an ordinary process thus PCR can be easily implemented (1)	Applied biosystem (1)	norganic method [proteinase K, salting out and chelex resin extraction method] (28)
Safety hood (4)	running cost of reagents and polymerase (1)	Biorad (3)	silica–gel membrane based absorption method [solid phase extraction] (13)
HVAC UV (6)	lack of armamentarium (3)	Invitrogen (1)	**TOTAL = 75**
Hypochlorite (18)	high rate of contamination (1)	Onelambda (2)	
RNase / DNase (4)	insufficient space (2)	Penicon (5)	
HEPA (2)	lack of technician (4)	Qiagen (20)	
Acetone (1)	power outage (2)	Roche (13)	
TOTAL = 58	difficulty in maintenance (1)	Sigma (5)	
	TOTAL = 36	Thermofisher (16)	
		Worldwidescientific (8)	
		TOTAL = 84	

DISCUSSION

In recent times, there has been rapid development of different types of PCR for detection of various pathogens.[11] For instance, Lochman et al. applied a real-time multiplex PCR for the identification of an array of cariogenic and periodontal bacterial species. Other less precise, semi-quantitative methods such as cultivating bacteria on media have been less productive in the past.[12] An additional advantage of Multiplex PCR detects DNA sequences of more than one pathogen simultaneously.[12] Rasool et al., an author of our study, previously utilized conventional PCR to discern 33% positivity of oral squamous cell carcinoma samples for exon 5 & 6 mutations of p53 gene.[13] Conventional PCR is performed to detect the presence of a specific DNA sequence. Meanwhile, the qPCR not only detects but also quantifies DNA sequence.[10] El-Shiekh et al. successfully detected and quantified high risk HPV genotypes 16, 18, and 31 (entailing E6 and E7 regions) in treatment-negative breast cancer by qPCR.[14] While qRT-PCR can be utilized to detect and quantify the expression level of mRNA. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines has shown to improve the specificity and sensitivity of qRT-PCR reactions.[15] Liu et al. utilized qRT-PCR to vindicate the results of microarray by comparing expression profiles of long non-coding RNA and mRNA between peri-implantitis and periodontitis.[16] In our study, 70% of the participants used other variants of PCR in addition to the conventional PCR, while 34% stated that conventional PCR is sufficient for their work. All of the aforementioned techniques are of prime importance in basic science and translational research due to high sensitivity and specificity in detecting and quantifying nucleic acid sequences. [12]

For a PCR to be performed the DNA needs to be isolated and separated from protein, cell membrane and other constituents of a lysed cell. DNA extraction methods are performed to purify

and separate DNA from aforementioned molecules.[10] For a DNA extraction method to be successful, it has to fulfill the following criteria: 1) minimum shearing; 2) high DNA purity ensued by absence of impurities during gel electrophoresis; 3) high yield typifying high double stranded DNA (dsDNA) quantity; 4) the availability of crude DNA as a DNA template for the PCR.[17]

Some of the common DNA extraction methods are as follows: 1) organic method (phenol–chloroform); 2) nonorganic method (proteinase K, salting out and chelex resin extraction method); and 3) silica–gel membrane based absorption method (solid phase extraction).[10] Almost half of the participants (45%) in our study routinely use the silica-gel membrane based absorption method, while 36% use the organic phenol chloroform method and 17% prefer the chelex resin method for DNA extraction, respectively. Sui et al., in a comparative study of 4 DNA extraction kits, noticed that Promega Maxwell gDNA (magnetic beads based silica method) and phenol:chloroform were associated with the highest DNA yield although it should be noted that the latter was the only method which developed impurities in negative controls. Promega also showed the least amount of bias in the process of DNA extraction.[18] Generally, the "phenol–chloroform" is a cheap yet labor intensive, multi-step DNA extraction method which produces high quality yield.[18] "Chelex" is a simple, one-tube extraction method which produces single stranded DNA. "Silica" method encompasses absorption of DNA with Silica beads or gel.[10] The importance of the selection of an appropriate DNA extraction method can be elucidated by the findings of Sui et al., Stinson, Keelan and Payne, and Videnska et al., who based on their findings posited that the decision to select a particular DNA extraction method had a profound impact on precision in identifying the microbial profile of fecal samples.[18, 19, 20]

In our study, almost half of the participants (42.5%) preferred to design their own primers. Numerous free online primer design software which are capable of designing good SYBR® Green-

based qPCR primers eliminate the need for laborious and time consuming calculations by humans and replace them by efficient algorithms.[21] Green and Sambrook posited that an appropriate primer design should entail 20-25 nucleotide bases, 40-60% G-C content, and a G or C nucleotide at the 3 end of the primer to avoid "breathing" of the ends of the primer.[5] Gupta posited that the ideal primer length should be 18-25 nucleotides.[10] The appropriate melting temperature (Tm) for primers has been suggested to be no less than 5 ℃ difference between both the members of a primer pair. Furthermore, it has been suggested that annealing temperature should be less than 3°C–5°C of the melting temperature of the primers.[5] Gupta purported that the annealing temperature should be 50–70°C.[10] The "nearest-neighbor thermodynamic" models are highly recommended for (Tm) calculation, although the "Wallace Rule" and the "Baldino algorithm" are easier to use and apt for most situations.[5] The characteristics of the PCR amplicon being produced is affected by the type of DNA polymerase selected for the cycle. For instance, Pfu polymerase is preferred over Taq polymerase if fidelity is desired, however, the latter is better in terms of processivity and cloning of a PCR product within the TA vector. Many manufacturers abet the client in the selection process of the DNA polymerase by providing properties with regards to the yield, fidelity, the choice of G-C rich amplification, speed and the desired target length. More importantly, now, several manufacturers put a blend of 2 or more thermostable DNA polymerases for a single PCR reaction. This enhances the validity and quality of the reaction.[5] Most of our participants (83.6%) prefer Taq polymerase provided by the manufacturer itself over other commercial variants. However, this choice of our participants didn't seem to influence the PCR performance ($p > 0.05$).

Green and Sambrook purported that at least 25 cycles are needed to obtain sufficient amplification of single-copy target sequence in DNA templates of mammals, while Gupta posited 30-40 cycles

for a conventional PCR run.[5,10] However, it should be pointed out that abounding number of cycles can lead to accumulation of unwanted secondary PCR product.[5] A conventional PCR run encompasses three main phases: 1) "Denaturation", which is characterized by a rise in temperature to about 94 °C - 95°C (depending on the G-C content of the template DNA, and the ramp rate) for 45 seconds; 2) "Annealing" conditions can be finalized by conducting mock PCR runs 2°C - 10°C lower than the melting temperatures of the primers; and 3) "Extension" (dependent on the DNA polymerase selected for the PCR run, for instance, Taq DNA polymerase amplifies the first 2kb of the primer at 70 °C to 80 °C in 1 minute, while Pfu DNA Polymerase amplifies 1 kb at 75 °C in 2 minutes.[5]

The participants of our study preferred to set the denaturation, annealing and extension temperatures of their PCR runs at 93 °C (±6.4), 55 °C (±4.6) and 71 °C (±5.4), respectively. It's worth noting that the temperature of all the 3 phases of a PCR cycle may be modified to optimize formation of the desired PCR product.[5] Forty two percent of our participants supported and 46% negated this philosophy of temperature alteration in PCR cycles. Interestingly, the decision of our participants to alter the temperature of the phases of PCR cycle had a very significant association (p 0.001) with the belief to either follow or not to follow manufacturer's protocol.

Spectrophotometry and gel electrophoresis can be utilized to discern the quality and yield of DNA.[10] Agarose gel utilized for electrophoresis contains 1% agarose mixed with 1× tris acetate EDTA or tris borate EDTA buffer. Agarose powder needs to be boiled for it to melt and completely mix with aforementioned buffers. Ethidium bromide (0.5 μg/ml) is also inseminated into agarose for the DNA to be viewed via a UV light and photographed at the end of the electrophoresis run. Initially, the agarose is placed in a water bath at 55°C. Once cooled, the molten agarose is poured in a gel mold containing combs. Around 0.5 to 1 mm of agarose should be present between the

base of the comb and the bottom of the gel mold.[22.] The respondents of our study set the mean thickness of the agarose gel to around 4 mm**.** Such a measure is taken to make sure that the sample wells are not porous and the agarose does not shred when the comb is removed.[22] More than half of our respondents (55%) alter the thickness of the gel when required.

Once the agarose settles at room temperature, the comb is removed. Then bromophenol blue is added to the DNA samples as a tracking dye. Once the voltage is at the recommended level of 1 to 5 V/cm of gel (the fragment size is dependent on the voltage chosen), the DNA samples with tracking dye are placed in the wells of the mold and electrophoresis is initiated. It is recommended to have two types of DNA size standards (low- and high-molecular weight) in the DNA samples.[22] The respondents of our study usually set a mean number of 20 wells for their gel electrophoresis runs. The negatively charged DNA fragments start moving to the positive lead. Bromophenol blue migrates simultaneously with the DNA fragments and the distance it travels serves as an indicator of the point at which DNA fragments separate. At this point, the electrophoresis can be switched off.[22] Nowadays, minigels (small gels) can be utilized to expedite the same process with higher throughput, higher reproducibility and lower cost as compared to larger conventional gels. Although, minigels are recommended for the analysis of DNA fragments of less than the size of 3kb.[22]

Majority of our respondents (86%) stated that choosing the right brand of PCR kit is pivotal to ensure adequate sensitivity and specificity of the PCR, and that different brands have varied PCR performance. Table 4 enlists the brands which are commonly chosen by our participants for performing PCR. The issue regarding the selection of the correct PCR kit brand came into limelight recently on a global scale during abounding use of RT-PCR for the detection of SARS-COV-2 infection. The U.S Food and Drug Administration (FDA) provided guidelines for test validation

to manufacturers during the SARS-COV-2 pandemic. Manufacturers have also started to use the MIQE guidelines for their experimental PCR runs to improve the quality of their products.[15] FDA also instructed the manufacturers to appraise the analytical and clinical test performance of their kit.[23, 24] Qiagen, as a brand, was selected most commonly (n = 20) by our respondents for the selection of their PCR kit including for DNA extraction. Sui et al noted that phenol: chloroform and Promega (magnetic beads based solid phase DNA extraction) methods gave the highest yield, but the former method had the highest contamination.[18] Stinson et al observed that Qiagen MagAttract PowerMicrobiome kit utilizing the same magnetic beads as Promega isolated highest microbial DNA from meconium samples.[19]

CONCLUSION

Our study's aim was to provide an insight into the preferences and practices of highly-qualified laboratory professionals working in prominent educational and research institutions of Karachi with regard to an important but relatively less understood tool called PCR. We also elucidated the workings of a qualitative (conventional) PCR in light of internationally accepted guidelines / standards. Our observations and recommendations will abet relatively new researchers in developing a working knowledge of this esoteric biological tool.

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